Pure Populations of Astrocyte Restricted Precursor Cells and Methods for Isolation and Use Thereof

Introduction

This application claims the benefit of priority from U.S. Provisional Application Serial No. 60/351,036, filed January 23, 2002, which is herein incorporated by reference in its entirety.

Field of the Invention

The present invention relates to a homogeneous, pure population of mammalian astrocyte restricted precursor cells which are CD44 immunoreactive and generate astrocytes but not oligodendrocytes. The present invention is also related to methods for isolating a homogeneous, pure population of these mammalian astrocyte restricted precursor cells. In addition, the present invention relates to use of mammalian astrocyte restricted precursor cells in the development of new transplantation techniques and to enhance myelination and/or reduce necrosis and glial scar formation upon administration to animals. The astrocyte restricted precursor cells and pharmaceutical compositions comprising the same, may thus be used to treat disorders of the nervous system resulting from trauma or disease which have in some way damaged the nerve tissue. These cells are also useful in identifying mammalian genes specific to selected stages of development.

Background of the Invention

Neural development has been well characterized in rodents. Multipotent cells which are nestin immunoreactive and capable of differentiating into astrocytes, neurons,
5 and oligodendrocytes have been identified by multiple investigators at various stages of development. In addition to multipotent precursors, other more restricted precursors have also been identified. Different populations of cells can be distinguished by differences in culture
10 conditions, self-renewal capability, as well as in their ability to integrate and to differentiate following transplantation.

Similar studies using human tissue are indicative of the existence of multiple types of neural precursors as
15 well. Multipotent human neural stem cells (hNSCs) have been isolated from fetal and adult tissue (Chalmers-Redman et al. Neuroscience 1997 76:1121-1128; Svendsen et al. J. Neuroscience Methods 1998 85:141-152; Vescovi et al. Exp. Neurology 1999 156:71-83; Carpenter et al. Exp. Neurology
20 1999 158:265-278; Quinn et al. J. Neuroscience Res. 1999 57:590-602; Piper et al. J. Neurophysiology 2000 84:534-548). These cells give rise to glia and neurons, can be grown under different culture conditions, and show different growth factor requirements.
25 Human neuron restricted precursors have also been described (Piper et al. J. Neurophysiol. 2000 84:534-548). Piper et al. used E-NCAM immunoreactivity to isolate neuronal precursor cells while Goldman and colleagues used neuron specific promoters to isolate neuronal precursors
30 (Roy et al. Nat. Med. 2000 6(3):271-7; Roy et al. J. Neurosci. Res. 2000 59(3):321-31; Wang et al. Dev. Neurosci. 2000 22(1-2):167-76). Human neuronal restricted precursor cells have been isolated from the adult ventricular zone and hippocampus as well as from fetal
35 tissue at multiple stages of development. These cells

differ from human neuroepithelial cells by their expression
of early neuronal markers such as NCAM, alpha-1 tubulin and
beta-III tubulin.

Proliferative adult human oligodendrocyte precursors
5 have been isolated from adult human white matter (Prabhakar
et al. Brain Res. 1995 672(1-2):159-69, Raine et al. Lab.
Invest. 1981 45(6):534-46; Scolding et al. Neuroreport 1995
6(3):441-5; Scolding et al. Neuroscience 1999 89(1):1-4)
using cell surface markers. Others have used
10 promoter-reporter constructs to isolate oligodendrocytes
and their precursors from fetal and adult tissue. A2B5
immunoreactivity has been utilized to isolate glial
precursors that are capable of differentiating into
astrocytes and oligodendrocytes (U.S. Patent 6,235,527).

15   Quinn and colleagues (J. Neurosci. Res. 1999 57:590-
602) describe a mixed population of multipotent stem cells
that can become altered in their properties after prolonged
culture. These cells have been suggested to be astrocyte
restricted precursor cells. However, oligodendrocyte
20 differentiation has not been tested. Further, no
information on antigenic expression, cytokine dependence,
response to growth factors, expression of GFAP/S100β, or
A2B5 is available. The cells of Quinn et al. were obtained
by sequentially passaging multipotent stem cells from
25 cultured human spinal cord tissue.

A putative astrocyte precursor cell has also been
described by Barres et al. (J. Neurosci. 1999 19(3):1049-
61). This cell was isolated from the optic nerve and its
existence in any other part of the brain is unknown. This
30 cell is A2B5 immunoreactive and thus resembles the
oligodendrocyte precursor O2A. The cells can be
distinguished from the O2A cells mainly by their failure to
develop into oligodendrocytes under conditions in which the
O2A cells readily generate oligodendrocytes. This cell is
35 Pax-6-positive and dies when exposed to serum.

Immunoreactivity with CD44 is unknown.

       Siedman et al. (Brain Res. 1997 753(1):18-26) have
also described an astrocyte cell line derived by
immortalization of a glial precursor cell. Little
5 information on this immortalized precursor cell is
available and its antigenic characteristics and ability to
differentiate into neurons have not been disclosed.

   Summary of the Invention
10   The present invention is directed to astrocyte
restricted precursor cells, pharmaceutical compositions
comprising the same, and methods of utilizing the astrocyte
restricted precursor cells to treat mammals with damage to
the nervous systems. The astrocyte restricted precursor
15 cells of the present invention are not immortalized. These
cells do not express A2B5. Further, these cells differ
from stem and progenitor cell populations in their
expression of CD44 and their ability to differentiate into
astrocytes under conditions in which other populations
20 differentiate into neurons or oligodendrocytes.

       Thus, one aspect of the present invention relates to
an isolated, pure homogeneous population of mammalian
astrocyte restricted precursor cells which is CD44
immunoreactive and can generate astrocytes but not
25 oligodendrocytes.

       Another aspect of the present invention relates to a
method for isolating a pure homogeneous population of
mammalian astrocyte restricted precursor cells. In the
method of the present invention, the pure homogeneous
30 population of astrocyte restricted precursor cells is
isolated from a heterogeneous or mixed population of
mammalian cells via CD44 immunoreactivity.

       Another aspect of the present invention relates to
methods for development of new transplantation techniques
35 using these mammalian astrocyte restricted precursor cells.

Another aspect of the present invention relates to pharmaceutical compositions comprising the mammalian astrocyte restricted precursor cells and methods of using these compositions to treat patients with damage of the

5   nervous system. In one embodiment, the compositions and methods are used to enhance myelination of mammalian neuronal cells. In another embodiment, the compositions and methods are used to reduce glial scar formation and necrosis.

10   Another aspect of the present invention relates to methods for identifying mammalian genes specific to selected stages of development using these astrocyte restricted precursor cells.

15 **Detailed Description of the Invention**

For cell replacement in the nervous system, differentiated cells are ultimately required. However, extensive studies have shown that differentiated cells do not survive well following transplantation. Therefore,

20 some researchers have focused their efforts on use of precursor cells which have been shown to survive and integrate into the intact or damaged brain.

The present invention relates to a pure, homogeneous population of mammalian astrocyte restricted precursor

25 cells which can be isolated from various sources of mammalian neural tissue and/or cells including, but not limited to, mammalian embryonic or fetal tissue, mammalian embryonic stem (ES) cell cultures, and glial restricted precursor cells. The present invention also relates to

30 methods for isolating a pure, homogeneous population of astrocyte restricted precursor cells from such tissues and cells.

For purposes of the present invention, by "pure" it is meant a population of cells in which greater than 95%,

35 more preferably 99%, exhibit the same characteristics.

In a preferred embodiment, the mammalian tissue or cells from which the astrocyte restricted precursor cells are isolated is either rodent or human. However, as will be understood by those of skill in the art upon reading

5   this disclosure, the methods for isolation taught herein are also routinely adaptable to cells or tissues from other mammals including, but not limited to, non-human primates, equines, canines, felines, bovines, porcines, ovines, lagomorphs, and the like

10   As demonstrated herein, the astrocyte restricted precursor cells of the present invention express CD44. Prior to differentiations these cells also express nestin. Unlike the putative astrocyte restricted cells of Barres et al. (J. Neurosci 1999 19(3):1049-61), the astrocyte

15 restricted precursor cells of the present invention do not express A2B5. Nor do the cells of the present invention express PSANCAM. The cells of the present invention grow well in FGF and EGF. The CD44-positive cells of the present invention do not express GFAP, vimentin or S-100β

20 initially, but have the capacity to differentiate into GFAP, vimentin and/or S-100β positive cells. Upon differentiation, the cells of the present invention maintain their CD44 immunoreactivity but lose expression of nestin. Thus, the CD44 positive cells of the present

25 invention can be readily distinguished from glial-restricted precursor cells, multipotent stem cells, neuronal precursors and the putative astrocyte precursor described by Barres et al. (J. Neurosci. 1999 19(3):1049-61) based on antigen expression, cytokine dependence and

30 differentiation ability. See Table 1 which provides a comparison of characteristics of the cells of Barres et al with the astrocyte restricted precursor (ARP) cells of the present invention.

TABLE 1		
Characteristic	Barres et al.	ARP Cells of Present Invention
A2B5 Expression	++	--
CD44 Expression	n.d.	++
Pax-6 Expression	++	--
Clonal analysis	n.d.	++
Transplant experiments	n.d.	++
Serum Exposure	death	differentiation

n.d. = not determined

The astrocyte restricted precursor cells are present in the developing mammalian brain prior to acquisition of GFAP immunoreactivity. In addition, the CD44+ astrocyte restricted precursor cells can be generated from glial-restricted precursors (GRP) and can be distinguished from GRP cells by antigen expression, cytokine dependency and differentiation ability.

Clonal analysis indicates that a subset of nestin+ cells that are GFAP- when grown in culture differentiate solely into astrocytes. This subset is quite large and constitutes approximately 11% of the cells analyzed.

A variety of markers were examined to identify a cell surface marker that would label this nestin+/GFAP⁻ population of cells. It was found that CD44 is specific for this glial population. CD44 expression co-localized with astrocytic markers such as GFAP and S-100β. CD44+ cells were RC1 negative and did not co-express A2B5. A small subset of the CD44+ cells were nestin immunoreactive but GFAP negative, thus indicating that these cells represented an astrocyte precursor cell population.

While the number of CD44 positive cells is small,

generally in the range of 1-10% of the total number of
cells present at any stage of development from E15 to
adult, it increases after culture in conditions that
promote astrocyte differentiation. CD44 positive cells
5 divide in culture and express low levels of GFAP.
Expression of GFAP increases after differentiation while
the expression of CD44 is down regulated. CD44 positive
cells do not express A2B5 or PLP and, thus, can be
distinguished from the bipotential glial-restricted
10 precursor cell. While CD44 expression has been described
in other cell types such as macrophages and astrocytes
following injury, under the differentiation conditions used
herein, CD44 expression was limited to astrocytes and,
thus, can be used in accordance with procedures taught
15 herein to identify astrocyte restricted precursor cells.
        Thus, as demonstrated herein, CD44 expression can be
used to identify and isolate astrocyte restricted precursor
cells from various sources of neural tissue including, but
not limited to, mammalian ES cell cultures and mammalian
20 fetal or embryonic tissue as well as glial-restricted
precursor cells or GRPS methods for isolating glial
restricted precursor cells are described in U.S. Patent
6,235,527 the teachings of which are herein incorporated by
reference in their entirety. This population of astrocyte
25 restricted precursor cells is not immortalized. Further,
population of cells does not express A2B5 and differs from
stem and progenitor populations in its expression of CD44
and its ability to differentiate into astrocytes under
conditions in which other populations differentiate into
30 neurons or oligodendrocytes.
        Various methods for isolating the CD44 positive
astrocyte restricted precursor cells from mixed populations
of cells can be used.
        In one embodiment, mammalian neural tubes are
35 dissociated at a stage after astrocyte development, for

example week 10 onward in humans or after E16 in rodents,
and dissociated cells are triturated to a single cell
suspension and labeled with an anti-CD44 antibody. Labeled
cells are visualized using a fluorescently labeled
5 secondary antibody targeted to the first antibody and
labeled cells are isolated using a selection process.

　　　　Examples of selection processes useful in the present
invention include, but are not limited to immunopanning,
magnetic bead sorting and/or FACS sorting. Detailed
10 magnetic bead and FACS sorting protocols are well known in
the art and can be routinely adapted to use of CD44 as the
selection marker. Further, as will be understood by those
of skill in the art upon reading this disclosure, negative
as well as positive selection methods can be used. Thus
15 enrichment of the astrocyte restricted precursor cells of
the present invention can be achieved by reselecting from a
mixed population cells that express CD44 but do not express
A2B5 or E-NCAM and vice versa. Positive and negative
selection processes can be used in any sequence and
20 antibodies with a binding profile similar to A2B5 or E-NCAM
can be used.

　　　　In another embodiment, neural tubes are dissociated
at any stage after neural tube closure, for example E8.5 in
mouse, E10.5 in rat, and week 5 gestation in human, and
25 cells are maintained in adherent culture for 5 to 40 days.
Cells are then removed from culture and CD44 positive cells
are isolated via a selection process as described in the
preceding paragraph.

　　　　In another embodiment, A2B5+ cells are isolated.
30 Cells are then induced to differentiate in culture by
growth in astrocyte promoting conditions. By astrocyte
promoting conditions it is meant to include, but is not
limited to, addition of bone morphogenetic proteins (BMPs),
oncostatin M, serum, Leukemia Inhibitory Factor/Ciliary
35 Neurotrophic Factor (LIF/CTNF) and other members of the

cytokine family such as interleukin-6 either singly or in
combination for a minimum period of three days. In a
preferred embodiment these agents are added at
concentrations in the range of 1-5 ng/mL. CD44+ cells are
5 then isolated via a selection process as described above.

Recently, human fetal tissue derived neural cells
have become available through commercial sources such as
Cambrex (East Rutherford, NJ), Clonexpress (Gaithersberg,
MD), ScienCell Research Laboratories (San Diego, CA) and
10 Clonetics (San Diego, CA). These cells serve as a source
of neural tissue and/or cells for isolation of the
mammalian astrocyte restricted precursor cells of the
present invention in accordance with the methods taught
herein.

15   Use of human ES cell lines as a source of the
astrocyte restricted precursor cells was also demonstrated
in three human cell lines (H1, H7, H9). Human ES cells
have been previously shown to differentiate into neuronal
progenitors that subsequently generate differentiated
20 neurons (Carpenter et al. Exp. Neurol. 2001 172(2):383-97).
In the present invention, dividing precursor cells that
expressed neuronal or glial markers were first identified
in ES cells. Differentiation conditions were similar to
those described herein and used for generating neurons.
25 Specifically, the first stage of differentiation of the ES
cells was induced by the formation of embryonic bodies
(EBs) in FBS media with or without 10 μM all trans-RA.
After 4 days in suspension, EBs were plated onto
fibronectin coated plates in defined proliferation media
30 supplemented with 10 ng/mL hEGF, 10 ng/mL hbFGF, 1 ng/mL
hPDGF-AA, and 1 ng/mL hIGF-1. In these conditions, the EBs
adhered to the plates and cells began to migrate and
proliferate on the plastic, forming a monolayer. After 3
days in these conditions many cells with neuronal
35 morphology were present. Similar results were found with

each human ES cell line.

Multiple types of dividing cell populations can be
identified in cultures of differentiating ES cells based on
antibodies that recognize cell surface epitopes. These

5 include A2B5+ cells, PSANCAM+ cells and CD44+ cells.
Double labeling experiments following differentiation
showed that the CD44+ cells of the ES cells were a unique
population of cells that were similar morphologically,
antigenically and in their ability to differentiate into

10 astrocytes to the astrocyte restricted cells of the present
invention isolated from other sources of neural tissue and
cells.

The astrocyte restricted precursor cells of the
present invention have a variety of uses.

15 For example, these cells can be used in nonhuman
mammalian models to develop new transplantation techniques.

In addition, these cells can be used therapeutically
in mammals, more preferably humans, in diseases
characterized by neural damage and more particularly

20 astrocyte degeneration. In particular, administration of
the astrocyte restricted precursor cells of the present
invention is expected to be useful in enhancing myelination
of neurons. These cells are also useful in identifying new
drugs which enhance survival and proliferation of these

25 cells upon administration.

The cells can also be used in the reduction of scars.
It is well known that fetal astrocytes can incorporate into
the brain when transplanted. Fetal cells, as opposed to
adult cells, reduce adult glial cell proliferation and scar

30 formation, thereby promoting repair. Astrocyte restricted
precursor cells of the present invention can be
administered at or near a lesioned site or area of damage
one week to several weeks after injury to reduce endogenous
adult glial cell proliferation and reduce scar formation.

35 Accordingly, the present invention also relates to

pharmaceutical compositions comprising these astrocyte restricted precursor cells for use in treatment of mammals with neural damage. In a preferred embodiment, the cells are provided in injectable form or on implants to promote

5   directed axon regeneration and reduce glial scar formation in the forebrain, and/or in damaged spinal axons of the central nervous system. Such compositions are useful in promoting CNS nerve regeneration and/or enhancing myelination and/or reducing glial scar formation.

10 Compositions comprising astrocyte restricted precursor cells can be applied, in various different formulations as described infra, to regions or areas of nerve damage. Such compositions can be administered to mammals having nervous system damage resulting from various causes including, but

15 not limited to, trauma, surgery, ischemia, infection, metabolic disease, nutritional deficiency, malignancies and paraneoplastic syndromes, toxic agents, and degenerative disorders of the nervous system. Examples of neurodegenerative disorders which can be treated using

20 compositions of the present invention include, but are not limited to, Alzheimer's disease, Parkinson's disease, Huntington's chorea, amyotrophic lateral sclerosis, progressive supranuclear palsy and peripheral neuropathies. Compositions comprising the astrocyte restricted precursor

25 cells of the present invention can also be applied to a wound to reduce scar formation. For example, following surgery, a composition comprising these cells can be applied in accordance with the presence invention to reduce scar formation from a lesion due to, for example,

30 arteriovenous malformation, necrosis, bleeding, and craniotomy, which can secondarily lead to epilepsy. The compositions of the present invention can also be used in the treatment of epilepsy by stabilizing the epileptic focus and reducing scar formation.

35   Pharmaceutical compositions of the present invention

comprise an effective amount of the isolated astrocyte restricted precursor cells of the present invention and a pharmaceutically acceptable carrier. By "effective amount" it is meant a composition comprising approximately 100,000

5 to about one million cells. As will be understood by one of skill upon reading this disclosure however, cell number may vary depending upon the selected site of administration. Examples of pharmaceutically acceptable carriers include, but are not limited to liquid vehicles

10 such as sterile saline, buffered saline, dextrose and water and semi-liquid or gel-like vehicles which may further comprise a media which impedes, at least in part, the mobility of the cells so as to localize the cells at the site of damage. Alternatively, pharmaceutically acceptable

15 carriers may comprise a solid vehicle such as an implant seeded or coated with the cells.

        The pharmaceutical compositions can be delivered by a wide range of methods to promote CNS nerve regeneration, enhance myelination and/or reduce scar formation.

20 Exemplary methods adaptable for use with the compositions of the present invention are set forth in U.S. Patent 5,202,120, the teachings of which are herein incorporated by reference in their entirety.

        In one embodiment, the cells are delivered by direct

25 application, for example, by direct injection of the cells in a vehicle into or near the site of nerve damage. In this embodiment, it may be preferred to deliver the cells in a vehicle comprising a media which impedes, at least in part, the mobility of the cells so as to localize the cells

30 at the site of damage. Examples of media which can impede cell mobility include, but are not limited, pastes or gels, such as biodegradable gel-like polymers of fibrin or hydrogels. These semi-solid medias also provide the advantage of impeding migration of scar producing

35 mesenchymal components such as fibroblasts into the site.

In another embodiment, the cells can be delivered via
a pharmaceutical compositions comprising a polymer implant
or using surgical bypass techniques. For example, the
astrocyte restricted precursor cells can be seeded or
5   coated onto a polymer implant. Various polymer implants
with differing composition, geometries and pore size which
can be used in this embodiment have been described.
Examples include, but are in no way limited to, implants
comprising nitrocellulose, polyanhydrides and acrylic
10 polymers. In a preferred embodiment, an implant with a
pore size of at least 0.45 μm is used.

The geometry of the implant is selected based upon
its intended use at the damage site. For example, an
elongated triangular implant may be selected to promote
15 nerve regeneration into the spinal cord dorsal root entry
zone while a pentagonal-shaped implant may be used to
promote nerve regeneration in the corpus callosum.

In another embodiment, the polymers may serve as
synthetic bridges over which nerve regeneration is promoted
20 and scar formation reduced by application of the astrocyte
restricted precursor cells at the ends or in the vicinity
of the ends of the synthetic bridge. For example, an
acrylic polymer tube with astrocyte restricted precursor
cells of the present invention at one or more ends, or
25 throughout the tube, can be used to bridge lesions
rostrally or bypass lesions, for example, of the spinal
cord, over which nerve regeneration can be induced.
Examples of such tubes are set forth in European Patent
Publication 286284, and in references by Aebischer et al.
30 (Brain Res. 1988 454:179-187 and Prog. Brain Res. 1988
78:599-603) and Winn et al. (Exp. Neurol. 1989 105:244-
250).

The cells of the present invention can also be used
in combination with surgical bypass techniques to promote
35 nerve regeneration and/or to reduce scar formation in a

selected region. Examples of such techniques which can be routinely adapted to use with the compositions of the present invention are set forth in U.S. Patent 5,202,120, which is herein incorporated by reference in its entirety.

5   The astrocyte restricted precursor cells of the present invention are also useful in the identification of genes specific to selected stages of development. In one embodiment, the cells can serve as a source of mRNA for generation of cDNA libraries that are specific to the stage

10 development of the cells.

The cells can also be used in the generation of cell lines and cell-specific antibodies for use therapeutically and diagnostically as well.

The following nonlimiting examples are provided to

15 further illustrate the present invention.

EXAMPLES

Example 1: Culture of Human Neural Stem Cells

Human neural progenitor cells derived from fetal tissue were acquired from Cambrex. Frozen aliquots of

20 cells were thawed and plated on fibronectin/laminin-coated multiwell dishes in Neural Progenitor Cell Basal Medium (NPBM, Cambrex) supplemented with human recombinant basic fibroblast growth factor, human recombinant epidermal growth factor, "neural survival factors", 5 mg/mL

25 gentamicin, and 5 mg/mL amphotericin-B (Singlequots, Cambrex). Cultures were incubated at 37°C, 5% CO_2 and fixed 24 hours later. These wells were subsequently processed for immunocytochemistry to assess the starting population of Cambrex cells. In parallel, Cambrex cells were thawed

30 and immediately plated on fibronectin/laminin-coated flasks (Greiner) and cultured in Neuroepithelial Precursor (NEP) medium that consisted of DMEM-F12 (Life Technologies) supplemented with additives as described by Bottenstein and Sato, basic fibroblast growth factor (bFGF, 10 ng/ml,

35 Peprotech, Rocky Hill, NJ), and chick embryo extract (CEE,

10%). Unattached cells typically formed floating spheres.
After 24 hours in culture, spheres were removed, gently
triturated, and re-combined with the attached cells. NEP
media was exchanged every other day.

5   Example 2: Isolation of Human Neuroepithelial Precursor
Cells (hNEPs)

    After 5 days in culture, immunopanning and
flow-activated cell sorting were used to remove ENCAM+,
NG2+, and A2B5+ cells. Briefly, cells were treated with 5
10 mM EDTA (Life Technologies) and the suspension plated on an
ENCAM antibody (5A5, Developmental Studies Hybridoma
Bank)-coated dish to allow binding of all ENCAM+ cells to
the plate. ENCAM antibody-coated dishes were prepared by
sequentially coating tissue culture dishes with an
15 unlabeled anti-mouse IgM antibody (10 mg/ml) overnight,
rinsing dishes with DPBS, followed by coating with 5A5
hybridoma supernatant for 1-3 hours at 37°C. Plates were
washed twice with DPBS prior to plating neural progenitor
cells. After a 30 minute exposure period, unbound cells
20 (eNCAM- cells) were removed and plated onto a dish coated
with antibodies to NG2 for 30 minutes. NG2 panning dishes
were made by coating dishes with an NG2 antibody (1:100)
for 1-3 hours at 37°C. The supernatant was then removed
(ENCAM-/NG2- cells) and immunostained for A2B5. Cells were
25 exposed to antibodies to A2B5 (1:2, Developmental Studies
Hybridoma Bank) in NEP media for 1 hour at 37°C, 5% CO_2. A
secondary goat anti-mouse IgM-PE labeled antibody was then
applied for 1 hour to stain the membranes of live A2B5+
cells. All cells were then sent through a flow-activated
30 cell sorter to remove the population of A2B5+ cells. After
sorting, the negative population (human NEPs) was
propagated in NEP media on fibronectin/laminin coated T-75
flasks prior to transplantation studies. NEP media was
exchanged every other day.

Example 3: Generation of Neurons, Oligodendrocytes, and
Astrocytes from hNEPs

Panned/sorted populations of human NEPs were plated
on fibronectin/laminin-coated 12mm coverslips in various
5 conditions to promote differentiation. To induce neuronal
differentiation, cells were exposed to bFGF (10 ng/ml) and
NT3 (10 ng/ml, Peprotech). After 5 days in culture, fixed
cultures were stained using antibodies to beta-III tubulin
to assess the capacity of these cells to differentiate into
10 neurons. For oligodendrocyte differentiation, cells were
plated in a bFGF (10 ng/ml)-containing medium for 2 days
and then were switched to a medium containing PDGF (10
ng/ml, Upstate Biotech., Waltham, MA) and T3 (50 nM) for 7
days. Antibodies to O4, GalC and MBP were used to identify
15 oligodendrocytes in culture. For astrocytic
differentiation, cells were cultured for 5 days in the
presence of fetal calf serum (10%, Life Technologies).
Astrocytes were identified using antibodies to CD44, GFAP
and S-100.

20 Example 4: Clonal cultures and clonal propagation

Mixed cell cultures of human fetal cells (12-22 weeks
of gestation) were obtained from Clonetics and plated in
T80 flasks in the presence of bFGF and CEE (10%). After 3
days in culture cells were labeled with A2B5 and NG-2.
25 Immunonegative cells were collected by FACS sorting
analysis and replated into flasks in the presence of
bFGF and CEE. After 24 hours cells were labeled with
E-NCAM, sorted by FACS and negative cells were replated at
a clonal density in 10 cm dishes in the presence of bFGF
30 and CEE. Control dishes were labeled after 24 hours with
A2B5, E-NCAM, GFAP and NG-2. At that time point, 97% of
all cells do not express any of the differentiation markers
tested. Single cells were grown at a clonal density of
50-200 cells/35 mm dish). Cells were maintained in FGF and
35 CEE for 8-10 days and then CEE was withdrawn to initiate

differentiation. For oligodendrocyte differentiation cultures were exposed to PDGF and thyroid hormone. After 5-7 days cultures were labeled with antibodies against GFAP and beta-III tubulin to determine differentiation into

5   astrocytes and neurons, respectively. Generation of oligodendrocytes was assessed 7 to 15 days after the initiation of differentiation. Neuronal and glial differentiation was assessed using antibodies against GFAP, and beta-III tubulin. For oligodendrocyte differentiation,

10 cultures were exposed to PDGF and thyroid hormone and differentiation was assessed using antibodies to O4 and Gal-C.

Example 5: Generation of Neurons, Oligodendrocytes, and Astrocytes from hNEPs

15   Panned/sorted populations of human NEPs were plated on fibronectin/laminin-coated 12mm coverslips in various conditions to promote differentiation. To induce neuronal differentiation, cells were exposed to bFGF (10 ng/ml) and NT3 (10 ng/ml, Peprotech). After 5 days in culture, fixed

20 cultures were stained using antibodies to beta-III tubulin to assess the capacity of these cells to differentiate into neurons. For oligodendrocyte differentiation, cells were plated in a bFGF (10 ng/ml)-containing medium for 2 days and then were switched to a medium containing PDGF (10

25 ng/ml, Upstate Biotech., Waltham, MA) and T3 (50nM) for 7 days. Antibodies to O4, GalC and MBP were used to identify oligodendrocytes in culture. For astrocytic differentiation, cells were cultured for 5 days in the presence of fetal calf serum (10%, Life Technologies).

30 Astrocytes were identified using antibodies to CD44, GFAP and S-100β (Morita et al. Dev. Neurosci. 1997 19:210-218; Gomes et al. Braz. J. Med. Biol. Res. 1999 32:619-631).

Example 6: Human ES cell culture

Male (H1) and female (H7 & H9) huES cell lines were maintained on MATRIGEL in MEF (primary mouse embryonic fibroblasts) conditioned medium (CM). CM was generated from huES cell media (ESM) comprised of 80% Knockout DMEM
5 (Gibco), 20% Knockout Serum replacement (Gibco), 0.1 mM beta-mercaptoethanol, 1 mM glutamine, 1% non-essential amino acids, supplemented with 4 ng/mL hbFGF (Gibco). Cultures were passaged by incubation in 200 units/ml collagenase IV (Gibco) for about 5-10 minutes at 37°C and
10 then gently dissociated into small clusters in CM. Cells were passaged about once every week. Conditioned media was generated from MEF and collected daily and used immediately for feeding HuES cultures. Before addition to the HuES cultures this conditioned media was supplemented with an
15 additional 4 ng/ml of hbFGF (Gibco). Cells for generating CM were refed with ESM daily and used for 7-10 days.

Example 7: Differentiation of huES cells

Embryoid bodies (EBs) were formed from undifferentiated ES cultures harvested by incubation with
20 200 u/mL collagenase at 37°C for 5-10 minutes. The cells were gently scraped from the dish and resuspended in ultra low attachment polystyrene plates (Corning) in media composed of KO-DMEM, 20% FBS, 1% non-essential amino acids, 1 mM glutamate and 0.1 mM beta-mercaptoethanol. In some
25 experiments, 10 μM all-trans retinoic acid was added to the EBs in suspension. After 4 days in suspension, EBs were plated onto poly-l-lysine/FN coated plates in proliferation media comprised of DMEM/F12 with B27 and N2 supplements (Gibco) and 10 ng/mL hEGF, 10 ng/mL hbFGF (Gibco), 1 ng/mL
30 hPDGF-AA (R&D Systems), 1 ng/mL hIGF-1 (R&D Systems). After 3 days in these conditions, the cells were harvested with trypsin and replated in differentiation media comprised of Neurobasal media supplemented with B27, 10 ng/mL hNT-3(R&D Systems) and 10 ng/mL hBDNF (R&D Systems).
35 These cultures were fed 3 times per week and fixed after

14-21 days.

Example 8: Immunocytochemistry

Cultures were stained using antibodies against A2B5 (1:2, Developmental Studies Hybridoma Bank), AC133/2

5   (1:100, Miltenyi Biotec, Auburn, CA), beta-III tubulin (1:1000, Sigma), E-NCAM (1:2, 5A5, Developmental Studies Hybridoma Bank), GFAP (1:2000, Dako, Carpinteria, CA), NG2 (1:100) and O4 (1:2, Developmental Studies Hybridoma Bank). Following fixation, cultures were treated with 0.5% Triton

10 X-100 (Sigma) in PBS for 2 minutes to access intracellular antigens. Fixed coverslips or plates were then treated with primary antibodies in a blocking solution containing Hank's balanced salt solution and 5% calf serum for 1 hour at room temperature. Following 3 washes with PBS, cultures

15 were incubated in the appropriate secondary antibodies (1:220) conjugated to either Texas Red or Alexa 488 (Molecular Probes, Eugene, OR) for 1 hour at room temperature. AC133/2 staining required amplification with a biotinylated secondary antibody, followed by a

20 streptavidin-alexa 488 conjugated tertiary antibody. All cultures were counterstained with DAPI (Molecular Probes) to identify cell nuclei.